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Role of TRPV4 in matrix stiffness-induced expression of EMT-specific LncRNA

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Abstract

Long non-coding RNAs (LncRNAs) are long (>200 bases), non-coding, single-stranded RNAs that have emerged as major regulators of gene expression, cell differentiation, development, and oncogenesis. In view of the fact that matrix stiffness plays a role in cellular functions associated with these processes, it is important to ask what role matrix stiffness plays in regulating expression of LncRNAs. In this report, we show that i) matrix stiffness causes differential expression of epithelial-mesenchymal transition (EMT)-related LncRNAs and mRNAs in primary mouse normal epidermal keratinocytes, ii) differential expression of EMT-related LncRNAs and mRNAs occurs in response to combined stimulation of transforming growth factor β 1 and matrix stiffness, and iii) transient receptor potential (TRP) channel of the vanilloid subfamily, TRPV4, a matrix stiffness-sensitive ion channel, plays a role in differential expression of EMT-related LncRNAs and mRNAs in response to combined stimulation by TGF β 1 and matrix stiffness. These data identify TRPV4 as a candidate plasma membrane mechanosensor that transmits matrix-sensing signals essential to LncRNA expression. Our results also show that we have established and validated an assay system capable of discovering novel LncRNAs and mRNAs sensitive to matrix stiffening.

Keywords

TRPV4; LncRNA; EMT; matrix stiffness; epithelial cells

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INTRODUCTION

Tissue stiffening is linked to numerous patho-physiological conditions including fibrosis, wound healing, oncogenesis, aging, and cardiovascular disease [1–8]. Pathogenesis of many stiffness sensitive conditions such as fibrosis and oncogenesis are characterized by an increase in cellular invasion and migration across a stiffened extracellular matrix (ECM) through induction of the epithelial-mesenchymal transition (EMT) program. EMT is a unique biologic process in which organized epithelial cells differentiate to motile scattered mesenchymal cells due to loss of cell-cell adhesions and apical-basal polarity leading to increased motility and invasiveness [5, 6]. EMT is essential in embryogenesis, oncogenesis, and wound healing, and aberrant regulation of this process can contribute to tumor invasion, metastasis, and tissue fibrosis [5–8].

Emerging studies showed that EMT is regulated by both a biomechanical signal, e.g., matrix stiffness, and a biochemical signal, e.g., transforming growth factor $\beta 1$ (TGF $\beta 1$) [9, 10]. Interestingly, it was shown that TGF $\beta 1$ requires a stiff substrate to drive EMT [7]. Furthermore, cells seeded on stiffer substrates with modulus mimicking the stiffness of fibrotic or tumor tissues, induced EMT and promoted invasion and metastasis [11, 12]. Previous reports from our laboratory and others have shown that the mechanosensitive ion channel, TRPV4, regulates matrix stiffness as well as TGF $\beta 1$ -induced signals to modulate cell differentiation and organ fibrosis [13–17]. Intriguingly, we recently showed that TRPV4 plays an important role in matrix stiffness as well as in TGF $\beta 1$ -induced EMT in both human and murine primary keratinocytes [18, 19]. However, the precise mechanism whereby TRPV4 mediates EMT is poorly understood.

TRPV4, a member of the TRP superfamily, is ubiquitously expressed in various cell types including keratinocytes [13–23]. Published work by our group and others have shown that TRPV4 is activated by a range of biochemical and biomechanical stimuli including changes in osmolarity, mechanical stress, and growth factors *in vitro* and *in vivo* [13–23]. TRPV4 has been linked to multiple physiological functions including osmolarity sensing in kidneys, and shearstress detection in blood vessels [13–23]. In mice, TRPV4 deficiency is linked to altered vasodilatory responses, osmosensing, and lung and skin fibrosis [13–23].

LncRNAs are long (>200 bases), non-coding, single-stranded RNAs that have emerged as major regulators of gene expression, cell differentiation, fibrosis, wound healing, development, and oncogenesis [24–26]. Genome-wide association studies have shown that genomic regions associated with human diseases contain LncRNAs, suggesting the therapeutic importance of LncRNAs in diseases, although the functional roles of most LncRNAs have not been fully determined [26]. Dysregulation in expression of LncRNAs have been linked to various cellular functions including EMT and the development of fibrotic diseases and cancers [24–34]. Since both ECM stiffening and aberrant LncRNA expression are associated with many patho-physiological responses, it is important to determine the role of matrix stiffening in expression of LncRNAs. Furthermore, the role of mechanosensitive TRPV4 proteins in matrix stiffness-induced expression of LncRNA has not been investigated. Here, we identify TRPV4 as a candidate plasma membrane mechanosensor that transmits matrix-sensing signals essential to LncRNA expression. Our

results also show that we have established and validated an assay system capable of discovering novel LncRNAs and mRNAs sensitive to matrix stiffening.

MATERIALS AND METHODS

Animal

Congenic wild-type (WT) C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA). TRPV4 knockout (TRPV4 KO) mice were generated on a C57BL/6 background by Dr. Suzuki (Jichi Medical University, Tochigi, Japan) [35] and were a generous gift from Dr. Zhang (Medical College of Wisconsin, Milwaukee, WI) [36]). The study was reviewed and approved by the University of Maryland review committee in compliance with the Institutional Animal Care and Use Committee (IACUC) guidelines on the care and use of animals. Mice were housed under pathogen free conditions with controlled temperature and humidity, and with food and water available *ad libitum*.

Primary normal mouse epidermal keratinocyte culture

Primary mouse normal epidermal keratinocytes (MNEKs) were harvested from the tail of 8–10 week-old WT and TRPV4 KO adult mice as described previously [18, 37]. MNEKs were maintained in dermal basal media supplemented with growth factors and penicillin/streptomycin (ATCC, Manassas, VA). Briefly, the mouse tail skin tissue was washed with sterile PBS and treated with trypsin (0.25%) for 2h at 37°C, 5% CO₂ to separate epidermis and dermis. The peeled epidermis was minced in cold dermal basal medium, triturated, strained, and resuspended in medium supplemented with growth factors (ATCC). Purity of MNEKs was examined by changes in morphology and by analyzing alterations of expression of epithelial/keratinocyte specific markers as previously published [18]. For the experiment, MNEKs were seeded on 100 mm collagen-coated polyacrylamide hydrogels (Matrigen Life Technologies; Brea, CA, USA) with soft (0.5 kPa) or rigid (12 kPa) stiffness followed by 24 h treatment with vehicle or TGFβ1 (5 ng/ml). TGFβ1 was purchased from R&D Systems (Minneapolis, MN, USA).

RNA isolation and quality control

Total RNA was harvested using the scraping method with TRIzol reagent (Thermo Fisher Scientific). Harvested RNA was purified with a RNeasy mini kit (Qiagen, Hilden, Germany) per the manufacturer's protocol, and quantified using the NanoDrop ND-1000. The OD₂₆₀/OD₂₈₀ ratios were inspected as an indication of purity.

Microarray analysis

The LncPath™ Mouse EMT Pathway LncRNA Microarray simultaneously profiles the expression of LncRNAs and their potential coding targets related to the EMT signaling pathway. Sample preparation and microarray hybridization were performed according to the manufacturer's protocol (version 5.7, Agilent Technologies, Arraystar, Rockville, MD). Briefly, LncRNAs/mRNAs were purified from total RNA after removal of rRNA using Arraystar rRNA removal kit. Each sample was amplified and transcribed into fluorescent cRNA along the entire length of the transcripts without 3' bias utilizing a random priming method. Labeled cRNAs were purified by RNeasy mini kit (Qiagen). Labeled cRNAs were

quantified by NanoDrop ND-1000. Later, labeled cRNAs were hybridized onto the 8×15 K LncRNA Mouse EMT Array (Arraystar). The microarray slides were incubated for 17 hours at 65°C in an Agilent hybridization oven. After washing, the hybridized arrays were fixed and scanned using an Agilent Scanner G2505C. Scanned images were imported into Agilent Feature Extraction software for raw data extraction.

Data analysis

Array images were extracted and analyzed using Agilent Feature Extraction Software (version 11.0.1.1). Quantile normalization and processing of data were performed using the R software limma package (Agilent Technologies). After quantile normalization of raw data, low intensity filtering was performed to retain the LncRNAs and mRNAs for further analysis (that at least 1 out of 5 samples have flags in 'present' or 'marginal'). LncRNAs or mRNAs having fold changes ≥ 1.3 were selected as significantly differentially expressed. Differentially expressed LncRNAs and mRNAs between the two groups were identified through scatter plot filtering.

RESULTS

Matrix stiffness modulates expression of EMT-specific LncRNAs and mRNAs in primary mouse normal epidermal keratinocytes

To assess the importance of matrix stiffness in LncRNA and mRNA expression, we obtained RNA from primary mouse normal epidermal keratinocytes (MNEKs) harvested from tails of 8 week-old wild type (WT) mice. MNEKs were seeded on collagen-coated 0.5 kPa (soft) or 12 kPa (stiff) polyacrylamide hydrogels. We used The LncPath™ Mouse EMT Pathway LncRNA Microarray (Arraystar, Rockville, MD) to profile the expression of ~ 200 LncRNAs related to EMT signaling pathway. Array images were extracted and analyzed using Agilent Feature Extraction Software. In this microarray analysis, we detected 101 LncRNA signals. A total of 26 LncRNAs showed significant differential expression ($p < 0.05$, fold change ≥ 1.3) (Figures 1A–C). These included 14 upregulated and 12 downregulated LncRNAs in high stiffness compared to low stiffness conditions. A total of 55 mRNAs were differentially expressed ($p < 0.05$, fold change ≥ 1.3). These included 32 upregulated and 23 downregulated mRNAs in high stiffness compared to low stiffness conditions (Figures 1D–F). Altogether, these results show differential expression of LncRNAs and mRNAs in response to matrix stiffness.

Differential expression of LncRNAs and mRNAs in response to combined stimulation of TGFβ1 and matrix stiffness

To determine the impact of combined TGFβ1 and matrix stiffness treatment in expression of LncRNAs and mRNAs, we treated MNEKs seeded on 12 kPa hydrogels with TGFβ1 (5 ng/ml). A total of 21 LncRNAs were differentially expressed (6 upregulated and 15 downregulated) in TGFβ1 plus high stiffness (12 kPa) treated cells compared to high stiffness alone (Figures 2A–C). A total of 69 mRNAs were differentially expressed (32 upregulated and 37 downregulated) in TGFβ1 plus high stiffness (12 kPa) treated cells compared to high stiffness alone (Figures 2D–F). These results show modulatory effects of TGFβ1 on expression of LncRNAs and mRNAs in response to matrix stiffness.

TRPV4 is a critical regulator of matrix stiffness-induced modulation of EMT-specific LncRNAs

To determine the role of TRPV4 in expression of LncRNAs, we seeded WT and TRPV4 KO MNEKs on 12 kPa hydrogels for 48 h and performed LncRNA analysis as above. Among 6 upregulated and 20 downregulated LncRNAs, we found 6 LncRNAs showing > 2-fold change ($p < 0.05$) in WT MNEKs compared to KO MNEKs (Figures 3A–C). A total of 58 mRNAs were differentially expressed (36 upregulated and 22 downregulated) in high stiffness TRPV4 KO vs high stiffness WT groups (Figures 3D–F). These data show that matrix stiffness-induced modulation of EMT-specific LncRNAs and mRNAs is dependent on TRPV4.

TRPV4 is a critical regulator in differential expression of LncRNAs and mRNAs in response to combined stimulation of TGFβ1 and matrix stiffness

Hierarchical clustering also revealed dysregulation in expression of LncRNAs and mRNAs among different treated groups (stiffness/TGFβ or stiff alone) (Figures 4A–C). Of 28 differentially expressed LncRNAs, we found 8 upregulated and 20 downregulated in the TGFβ1 plus high stiffness TRPV4 KO group compared to the TGFβ1 plus high stiffness WT group. Among 61 differentially expressed mRNAs, 31 were upregulated and 30 were downregulated (Figure 4D–F). These data show that matrix stiffness-induced modulation of EMT-specific LncRNAs and mRNAs is dependent on TRPV4.

DISCUSSION

During the progression of wound healing, fibrogenesis, or oncogenesis there is a significant contribution of fibrotic mesenchymal cells derived from the resident epithelial cell population due to EMT. In tissues, EMT causes transition of epithelial cells into fibroblasts and/or myofibroblasts, which further perpetuate wound healing, fibrosis, or oncogenic responses through the generation of exaggerated ECM and increased tissue stiffening. Although TGFβ1 is recognized as a primary contributor to the onset and progression of EMT, recent studies have shown that matrix stiffness alone or in combination with soluble factors such as TGFβ1 potently regulates EMT in various biological contexts [7, 11, 12, 38, 39]. The signaling mechanisms underlying EMT have been well studied; however, the identity of a matrix stiffness-sensing plasma membrane receptor and the molecular mechanisms by which matrix stiffness signals are transduced into cells to drive the EMT remain to be determined. LncRNAs have recently emerged as powerful regulators of gene expression in numerous cellular processes including cell differentiation, development, and lineage commitment [24–26, 40–42]. LncRNAs are transcripts that contain no open reading frame and lack protein coding capacity. Various modes of action of LncRNAs have been documented [24–26, 40–42] including acting as: (i) guides to recruit chromatin modifying enzymes to activate or repress gene expression; (ii) scaffolds bringing proteins into close proximity to form activation or repression complexes; (iii) decoys to sequester transcriptional or other regulatory factors such as microRNAs (miRNAs); and (iv) enhancers to increase expression of neighboring genes. Thus, it is important to determine whether expression of LncRNAs is influenced by degree of matrix stiffness. In this report, we show that 1) matrix stiffness causes differential expression of LncRNAs and mRNAs in primary

mouse normal epidermal keratinocytes, 2) differential expression of LncRNAs and mRNAs in response to combined stimulation of TGF β 1 and matrix stiffness, and 3) TRPV4 plays a critical role in differential expression of LncRNAs and mRNAs in response to combined stimulation of TGF β 1 and matrix stiffness. These data identify TRPV4 as a candidate plasma membrane mechanosensor that transmits matrix-sensing signals essential to LncRNA expression.

A recent report showed that LncRNA profiles were altered in TGF β -induced EMT in mammary epithelial cells [43]. Differentially expressed LncRNA and mRNA profiles have been reported in rat fibrotic lung tissues and in regressive and mature scars obtained from patients [44, 45]. Here, we report a differential LncRNA expression signature during stiffness-induced EMT-like transition with or without TGF β 1 in WT and TRPV4 KO primary keratinocytes. Among differentially expressed LncRNAs, we found that changes in the expression of AK050884, ENSMUST00000134701, TCONS_000000072, and ENSMUST00000141797 were associated with changes in expression of Ctnnd1, Ezh2, Wnt10a, and Cdkn1a mRNAs, respectively. There have been recent reports that LncRNA participate in TGF β -induced EMT in human and mouse epithelial cells, but so far, no report has shown an association of LncRNA and TRPV4 during stiffness-induced EMT-like transition [43, 46]. We recently showed that the mechanosensitive ion channel, TRPV4, contributes to TGF β 1- and matrix stiffness-induced EMT-like transition, myofibroblast differentiation *in vitro* in primary human and mouse cells, expression of mesenchymal markers, NCAD and α -SMA, in a bleomycin-induced murine skin fibrosis model, and development of lung and skin fibrosis [14, 15, 18, 19, 23]. Skin keratinocytes are the target of chronic inflammatory skin disorders, including psoriasis and systemic sclerosis that further leads to skin fibrosis. The increased EMT that contributes to skin fibrosis in an experimental mouse model [47], and in patients with systemic sclerosis [48] has been linked to the increased activation of TRPV4 in skin keratinocytes in a matrix stiffness-dependent manner [18, 19]. Since, skin keratinocytes express functional TRPV4, and undergoes EMT, we used skin keratinocytes as a model cell to establish and validate an *in vitro* assay system capable of discovering novel LncRNAs and mRNAs sensitive to matrix stiffening. For the analysis of LncRNAs and mRNAs, MNEKs were seeded on collagen-coated polyacrylamide hydrogels with soft (0.5 kPa) or stiff (12 kPa) stiffness followed by treatment with vehicle or TGF β 1. We selected this stiffness range to mimic stiffness of normal skin tissue (0.5 kPa) and fibrotic skin (~8–50 kPa) (49, 50). Our current results reveal differentially expressed LncRNAs and mRNA profiles in response to TGF β 1- and matrix stiffness-induced stimulation in TRPV4 KO keratinocytes compared to WT. These results suggest a link between TRPV4 and LncRNA expression in response to stiffness. These results also demonstrate that we have established and validated an *in vitro* assay system capable of discovering novel LncRNAs and mRNAs expressed in various pathophysiological conditions under increasing matrix stiffening.

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Figure 1. Differential expression of EMT-specific LncRNAs and mRNAs in primary mouse normal epidermal keratinocytes in response to matrix stiffness.

Hierarchical clustering and heatmap analysis shows distinguishable LncRNA and mRNA expression profiling between stiff and soft stiffness groups. Hierarchical clustering (A) and scatter plot (B) depicts the comparison of LncRNA expression between two groups; fold changes ≥ 1.3 are shown above and below the green lines. (C) List of up- or down-regulated LncRNAs in response to matrix stiffness. Low passaged (< 4) MNEKs were seeded on collagen-coated polyacrylamide gels with soft (0.5 kPa) or stiff (12 kPa) rigidity followed by 48 h incubation to induce an EMT-like response. Hierarchical clustering (D), heatmap analysis (E), and differentially expressed mRNAs (F) between stiff and soft stiffness groups.

Figure 2. Combined stimulation of TGFβ1 and matrix stiffness causes differential expression of LncRNAs and mRNAs.

Hierarchical clustering (A) and heatmap analysis (B) shows LncRNA expression profiling in stiff groups in the presence or absence of TGFβ1. (C) List of up- or down-regulated LncRNAs. Hierarchical clustering (D), heatmap analysis (E), and differentially expressed mRNAs (F) between stiff and soft plus TGFβ1 groups.

Figure 3. TRPV4 is a critical regulator in matrix stiffness-induced modulation of EMT-specific LncRNAs and mRNAs.

Hierarchical clustering and heatmap analysis shows differentially expressed LncRNA and mRNA expression profiling between WT and TRPV4 KO MNEKs maintained on stiff matrix. Hierarchical clustering (A) and scatter plot (B) shows the comparison of LncRNA expression between WT and TRPV4 KO cells; fold changes ≥ 1.3 are shown above and below the green lines. (C) List of up- or down-regulated LncRNAs in WT and TRPV4 KO cells. Hierarchical clustering (D), heatmap analysis (E), and differentially expressed mRNAs (F) between WT and TRPV4 KO cells maintained on stiff matrix.

Figure 4. Combined stimulation of TGFβ1 and matrix stiffness causes differential expression of LncRNAs and mRNAs in a TRPV4-dependent manner.

Hierarchical clustering (A) and scatter plot (B) depicts the comparison of LncRNA expression between WT and TRPV4 KO cells after combined stimulation with TGFβ1 and matrix stiffness. (C) List of up- or downregulated LncRNAs in response to both stiff matrix and TGFβ1. Hierarchical clustering (D), heatmap analysis (E), and differentially expressed mRNAs (F) between WT and TRPV4 KO cells after combined stimulation with stiff matrix and TGFβ1.